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PRINCIPAL INVESTIGATOR: Stephen L. Eck, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

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6. AUTHOR(S)

Stephen L. Eck, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246**8. PERFORMING ORGANIZATION
REPORT NUMBER****E-MAIL:**

ecks@mail.med.upenn.edu

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)U.S. Army Medical Research and Materiel Command
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The principal goal was to understand why breast cancer cells are able to evade the host immune system despite the presence of tumor antigens and tumor antigen-specific T lymphocytes. We had previously demonstrated that tumor-derived prostaglandin E₂ (PGE₂) directly contributes to the lack of a significant immune response to breast cancer cells. However, the production of PGE₂ by breast cancer cells did not completely explain the immune suppressive effect of breast cancer cells. We have subsequently demonstrated that GA733-2/mEGP, a type I cell surface breast cancer protein, is able to efficiently block the presentation of a variety of antigens from dendritic cells (DC). Murine DC expressing mEGP were unable to stimulate allogeneic T cell responses or responses to model tumor antigens. Using in vivo models, both B cell and T cells failed to respond to viral antigen presented in the context of mEGP. Additionally, we have shown that mEGP increases the activity of cathepsin, a protein believed to be involved in local tissue invasion and metastasis. These data, and the recent reports of poorer outcomes for women with GA733-2 expressing breast cancers, suggest that mEGP/GA733-2 may be a suitable target for therapeutic intervention.

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FOREWORD

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N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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PI - Signature

Date

10/15/01

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Human breast cancer derived PGE₂ inhibits B7-1 induced T cell proliferation

Key words: breast cancer, immunotherapy, immunosuppressive factors, GA733-2, mEGP, antigen presentation

INTRODUCTION

The principal goal of this study has been to understand why breast cancer cells are able to evade the host immune system despite the presence of tumor antigens and tumor antigen-specific T lymphocytes. At the outset we postulated that the production of prostanoids, principally prostaglandin E₂ (PGE₂), by the tumor directly contributes to the lack of an immune response to breast cancer cells. As reported previously, we found that human breast cancer cells secrete soluble agents that directly inhibit T lymphocytes and that one of the major inhibitory factors secreted by breast cancer cells is PGE₂. This demonstrated that an important function of PGE₂ is to directly alter or suppress the immune response to breast cancer cells. Although our data suggested that PGE₂ derived from human breast cancer cells contributes to inhibition of cellular immunity, it became apparent that other factors (not identified at the time of our proposal submission) played an equally important (if not greater) role in breast cancer mediated immune suppression. Last year we reported on our work that demonstrated the potential role of mEGP (murine homologue of GA733-2) in breast cancer mediated immune suppression. These murine and human tumor associated antigens are found on some but not all of murine and human breast carcinomas, respectively. A recent report in the past year showed that the expression of GA733-2 on human breast cancer cells independently contributed to a worse prognosis for women with all stages of breast cancer [1]. In the past year we have conducted additional experiments to refine our understanding of how GA733-2 and mEGP contribute to a poor prognosis and what potential implications this may have for therapies of breast cancer.

BODY OF REPORT

As noted in our prior report, the current line of investigation extended beyond that initially proposed. This was brought about by two circumstances. (1) We were not able to conduct some of the experiments originally proposed for technical reasons. The initial review group raised this concern in their evaluation of the proposed research and undoubtedly this contributed to their concern about the feasibility of parts of our proposed plan. (2) Our data had uncovered the contribution of mEGP/GA733-2, which had not been appreciated at the time the proposal was submitted. The structure and function of mEGP/GA733-2 was described in detail in the prior report and will not be restated here in detail. However, in summary they are type 1 transmembrane proteins believed to have adhesive functions in epithelial cells (both normal and malignant). We demonstrated that mEGP and GA733-2 had the immunologic activities as listed in Table 1 (see also attached manuscript under review at Nature Medicine).

Table 1. Summary of the immunologic Activities of mEGP
Blocks MHC class II restricted antigen presentation when expressed in dendritic cells (DC)
Inhibits antigen presentation of allogeneic antigens and defined antigens such as OVA and HEL
Inhibits T cell activation by DC when DC are exposed to tumor cell lysate containing mEGP
Human GA733-2 inhibits a human mixed lymphocyte reaction

The extracellular Domains of mEGP and GA733-2 contain the inhibitory motif.

The similarity of the GA733-2 and mEGP protein sequences and the expression of GA733-2 and mEGP on human breast and mouse mammary tumors, respectively, strongly suggested that the biologic behavior of the murine protein would predict the behavior of the human protein. This notwithstanding, the human protein GA733-2 did not inhibit murine DC transfected to express GA733-2. We postulated that the inhibitory activity of both proteins resided in the extracellular domain and that small differences in their short intracellular domain might account for their species specific behavior. This was supported by our observation that a truncated form of mEGP (mEGPex) that lacked the intracellular domain (contained only the transmembrane and extracellular (ex) domains) and GA733-2 (different intracellular domain) did not inhibit DC when transfected into DC (not shown, see attached manuscript). However, both mEGPex and GA733-2 could inhibit the DC when they were expressed in tumor lysate (see figure 1 below).

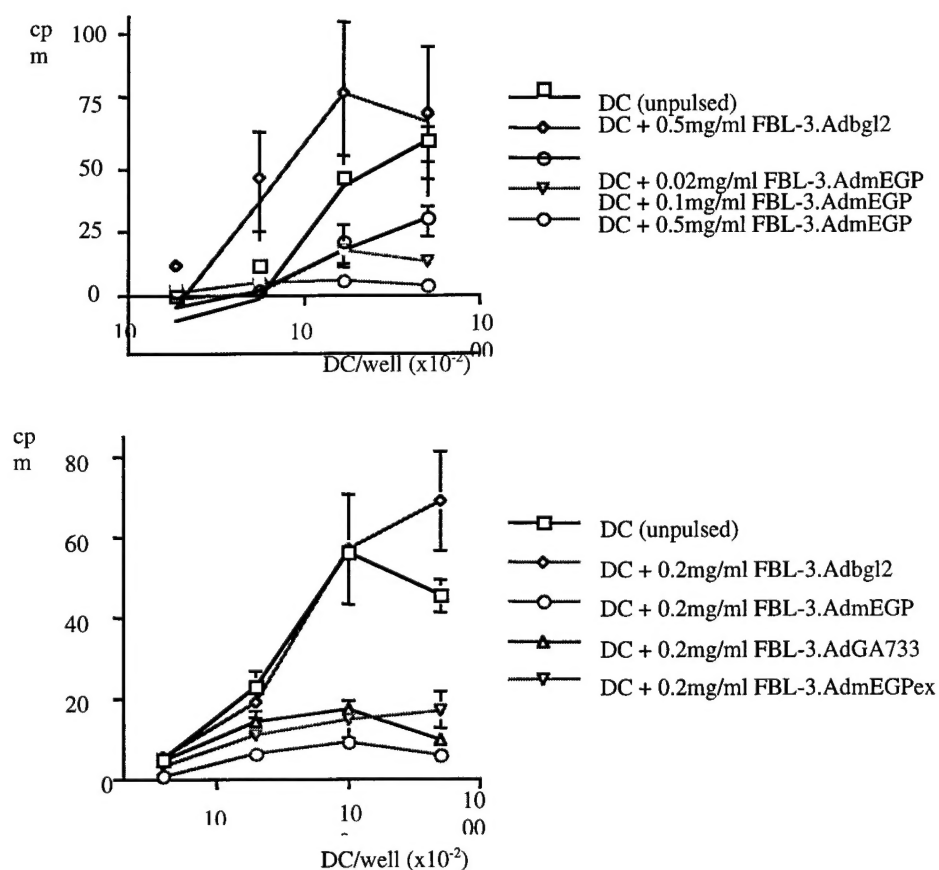


Figure 1 (above) DC pulsed with mEGP containing tumor cell debris lose their stimulatory capacity in an allogeneic MLR. DC were pulsed for 16h with different concentrations of cell debris containing mEGP (FBL-3.AdmEGP) or control cell debris (FBL-3.Adbgl2). They were then used to stimulate T cells in an allogeneic MLR. DC exposed to cell debris containing mEGP resulted in a dose dependent (top graph) decrease of T cell proliferation. Tumor debris containing mEGP, GA733-2 or mEGPex were equally effective in inhibiting a MLR (bottom graph) .

These data (see manuscript and prior report) suggest that mEGP and GA733-2 are able to inhibit antigen presentation and thereby may limit the ability of the patient's immune system to respond to unique breast cancer antigens. Our data (not shown) shows that naturally expressed GA733-2 (from SW480 cells) similarly inhibits T cells activation. In contrast, Hela cells that lack GA733-2 expression have no effect on DC activity. This observation underscores that the inhibitory activity of GA733-2 lies in the protein itself and is not an artifact of the transfection system. To the extent that mEGP and GA733-2 are expressed in select normal epithelial cells, we postulate that these proteins have evolved to block the immune response to normal host antigens that are not available for negative selection of T cells in the thymus. That is, mEGP and GA733-2 are likely part of the normal maintenance of peripheral tolerance. With respect to normal breast tissue, we postulate that proteins found in breast milk could serve as neoantigens were it not for the presence of a peripheral tolerance mechanism. We believe that EGP and GA733-2 fulfill this role of establishing peripheral tolerance. Breast tumors have evolved to co-opt this mechanism, and the poor prognosis of GA733-2 expressing breast tumors may be a consequence of a decreased immune response to breast tumor antigens. To further examine this issue we have begun to address whether the immune suppression seen in vitro can also be seen in vivo.

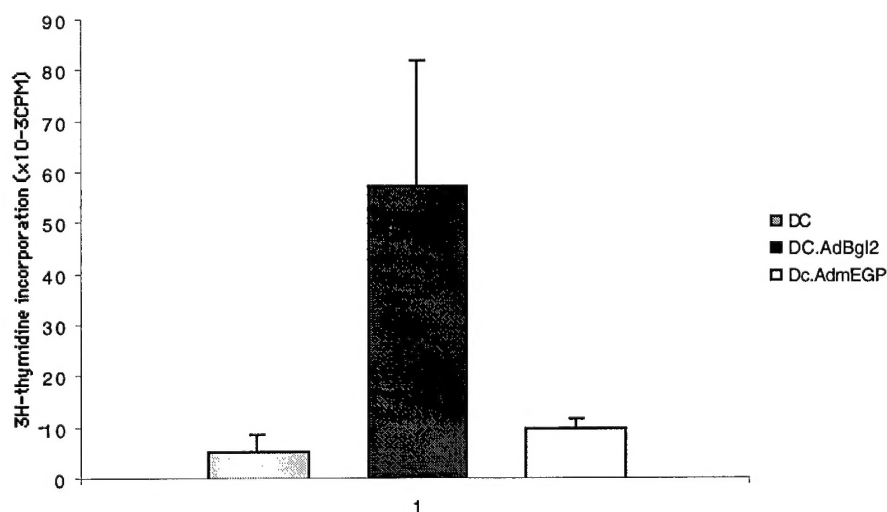
Dendritic cells expressing mEGP fail to mediate B and T cell responses to potent viral antigens.

To test this hypothesis we used autologous bone marrow derived dendritic cells (DC) transfected with either a control adenovirus (Ad.Bgl2) or an adenovirus expressing mEGP. These recombinant adenoviruses are non-replicating but are able to deliver a broad spectrum of highly immunogenic adenoviral antigens. Moreover, the DC are the most potent T cell activators. Following a single intravenous injection of transfected (Ad.Bgl2 or Ad.mEGP) DC, or untransfected DC (negative control) the mice were euthanized (one week later) and splenocytes examined for their ability to respond to adenoviral antigens. As seen in Figure 2, there is a marked reduction of T cell proliferation in response to adenoviral antigens in those mice receiving DC expressing mEGP and viral antigens compared to the mice receiving DC expressing only viral antigens. This observation indicates that mEGP is inhibitory in an in vivo antigen presentation context using highly immunogenic viral antigens.

Up to this point we had only examined the effect of mEGP and GA733-2 on T cells. However, B cell (antibody) response to tumor antigens may also be important as underscored by the therapeutic efficacy of trastuzumab (Herceptin®, an anti-HER2 monoclonal antibody) in the therapy of breast cancer. We postulate that in the absence of CD4 T cell responses (as demonstrated above and previously), antibody production would be markedly diminished. This is illustrated in Figure 3, where the same experimental design as Figure 2 was employed. Subsequent experiments reveal that these differences in antibody titer are maintained over time (examined out to day 28 so far). This indicates that it is not a delay in antibody production but rather a prevention of antibody development. Also of interest is the broad spectrum of antibody suppression. This suggests that both helper-dependent and helper-independent antibody production is suppressed. This implies that mEGP works by a mechanism other than (or in addition to) inhibition of CD4 T cell activation. As noted above we had initially postulated that antibody suppression might occur as a result of deprivation of CD4 help. However, recent work by others has suggested a more direct mechanism. LAIR-1 was previously cloned by homology to other lymphocyte receptors [2] and postulated to serve as an inhibitor of lymphocyte function based on evidence that it had phosphatase activity. While currently, there is no functional data on LAIR-1, it was recently shown that GA733-2 (also known as EpCam) is the ligand for LAIR-1. Moreover, the LAIR-1 binding portion of GA733-2 is confined to the distal portion of the extracellular domain. This is consistent with our observations of here the inhibitory activity lies.. Although no murine homolog of LAIR-1 is known,

these observations suggest that mEGP and GA733-2 may function by direct interaction with the lymphocytes. The broad distribution of LAIR-1 on T cells, B cells and NK cells provide further evidence of the potential potency of mEGP/GA733-2 engagement. Moreover, the critical domain of GA733-2 that interacts with LAIR-1 is the N-terminal EGF-like region. This provides an important clue for targeted drug development in this area.

Figure 2. DC expressing mEGP were markedly less efficient in vaccinating mice against co-expressed adenoviral antigens compared to Adbgl2 transfected control DC.



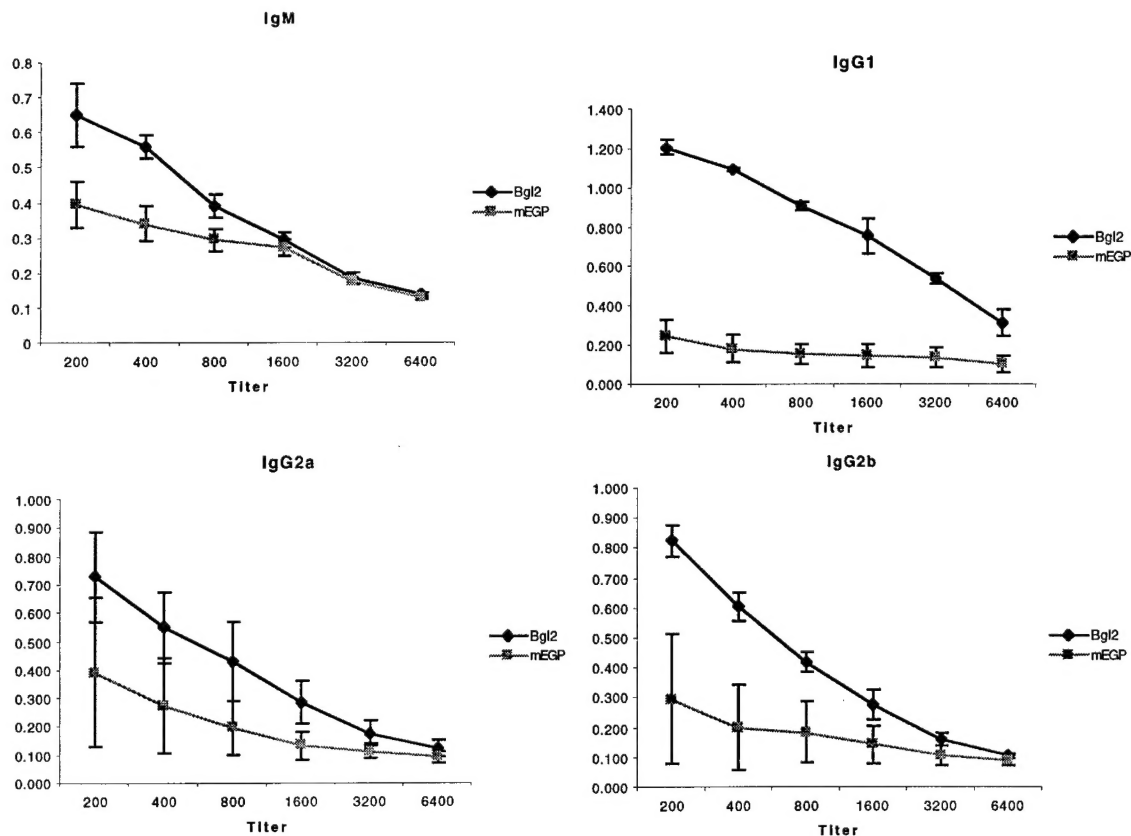


Figure 3. Anti-adenoviral antibody titers. Sera obtain 7 days after a DC injection were tested for anti-adenoviral antibodies by ELISA assay. Mice exposed to adenoviral antigens in the presence of mEGP had marked reduced antibody titers.

mEGP expression increases cathepsin S activity.

Previously we had identified the thyroglobulin domain (TGD) in mEGP as a potentially important domain for biologic activity. This was based on the known ability of TGD to bind to the cathepsin serine proteases, which are needed for antigen processing by DC and other antigen presenting cells. We therefore examine the effect of mEGP expression on cathepsin S activity in vitro. We transfected DC or RAW macrophage cells with Ad.mEGP and measured the changes in cathepsin activity. As shown in Figure 4, expression of mEGP markedly increases the cathepsin activity. The mechanism by which this occurs is not known and is under investigation. However, expression of tissue matrix proteases (including as cathepsins) has been associated with increases in micro-invasiveness and metastasis of many tumor types including breast cancers. We have recently prepared a deletion mutant of mEGP that lacks the TGD and are examining whether this domain is needed for the increase in enzymatic activity seen in figure 4. These studies may point to a second function of mEGP/GA733-2 that enables breast cancer progression.

Cathepsin Activity in RAW Macrophages - 7.17.01

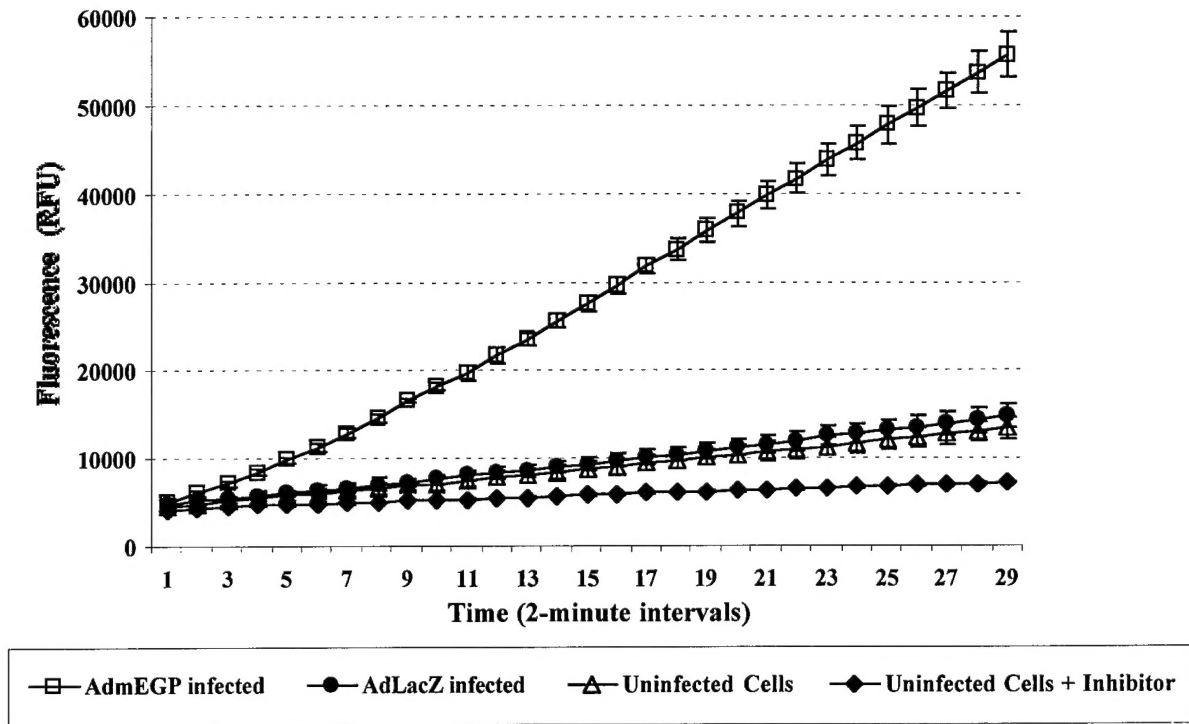


Figure 4 Cathepsin activity following mEGP expression. Cells were cultured in the presence of a fluorescent substrate specific for cathepsin S with or without a cathepsin inhibitor.

KEY RESEARCH ACCOMPLISHMENTS (entire project)

These studies demonstrate that:

1. Production of PGE₂ by breast cancer cells occurs at levels that can inhibit T cell activation in the tumor environment.
2. Indomethacin inhibits PGE₂ mediated T cell suppression.
3. The expression of cyclooxygenase (COX) and the resultant production of PGE₂ are sufficient to abrogate the T cell response to tumor cells in a vaccination model.
4. mEGP when ectopically expressed in DC blocks an allogeneic mixed lymphocyte reaction as assessed by T cell proliferation, IL-2 production and interferon- γ production.
5. Inhibition of T cell activation by mEGP is dose dependent, and exhibits no "trans" effect.
6. T cell activation in the MLR is restored in the presence of mEGP when Con A or anti-CD3 antibody is added to the MLR, however, antibodies to mEGP do not restore T cell responses.
7. mEGP blocks the response of lymphocytes with transgenic T cell receptors for OVA and HEL both when the intact protein is used as the antigen or when the specific class II restricted peptide is used as the antigen.
8. mEGP is able to block class II but not class I restricted presentation of OVA antigen.
9. mEGP when provided in the form of a lysed tumor cell expressing mEGP is also able to block T cell activation as assessed in both the MLR and OVA model experiments.
10. A truncated form of mEGP lacking the cytoplasmic domain is not able to block T cell activation when expressed in the BMDC but is able to block T cell activation when provided in tumor cell debris. This also holds true for the human antigen GA733.
11. mEGP does not alter BMDC morphology, cell surface expression of key T cell stimulatory molecules (e.g., B7-1, B7-2, class I, class II, CD 11b, CD 11c), production of IL-12 and overall viability.
12. Lysate from tumor cells expressing mEGP or GA733 blocks T cell activation.
13. In vivo administration of mEGP in antigen presenting cells results in suppression of T cell responses to potent viral antigens.
14. In vivo administration of mEGP in antigen presenting cells results in suppression of B cell responses to viral antigens.
15. IgM and all IgG subclasses are equally inhibited by mEGP suggesting a T helper cell independent inhibition.
16. The expression of mEGP leads to increase cathepsin activity that may enhance the ability of the tumor to invade stromal tissue.

REPORTABLE OUTCOMES

1. A manuscript has been submitted to Nature Medicine.
2. Our finding that proteins in the membrane of breast tumor can inhibit an immune response is the basis of a RO1 grant application to further pursue this finding. "Inhibition of T cells by a Breast Tumor Assoc. Antigen", NCI, Funding from this new grant will continue the work described here.

CONCLUSIONS

We have provided evidence that tumor-derived PGE₂, limits the immune response to breast cancer cells in an experimental model. In addition, certain membrane proteins in breast cancer cells (GA733-2 antigen) appear to block T cell and B cell responses by indirectly interfering with antigen presentation by professional antigen presenting cells. GA733-2 and mEGP may also have a direct effect on lymphocytes through binding of the putative inhibitory receptor LAIR-1. Finally, mEGP is able to substantially enhance cathepsin activity. The biologic and clinical significance of these findings is not yet established. However, they are in concert with the observations that GA733-2 expression in sporadic breast cancer contributes to their poor clinical outcomes. To that extent, drug development strategies may profitably target the pathways identified in this research project.

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APPENDIX CURRICULUM VITAE OF PRINCIPAL INVESTIGATOR

UNIVERSITY OF PENNSYLVANIA - SCHOOL OF MEDICINE

Curriculum Vitae

July 2001

Stephen L. Eck, M.D., Ph.D.

Office Address: Rm. 556, Biomedical Research Building II/III
The University of Pennsylvania
421 Curie Blvd.
Philadelphia, PA 19104-6160
(215) 898-4178, FAX: (215) 573-8590, pager 610-734-8226
email: ecks@mail.med.upenn.edu

Education: B.A. Kalamazoo College (Chemistry)
M.S. Harvard University (Chemistry)
Ph.D. Harvard University (Chemistry)
M.D. University of Mississippi School of Medicine

Postgraduate Training and Fellowship Appointments:

1981-1982 Senior Scientist, Monsanto Company, St. Louis, MO.
1982-1987 Res. Associate, Dept. of Biochemistry, Univ. Miss. School of Medicine, Jackson, MS.
1987-1988 Intern in Medicine, University of Michigan Hospitals, Ann Arbor, MI
1988-1989 Residency in Medicine, University of Michigan Hospitals, Ann Arbor, MI
1989-1992 Hematology/Oncology Fellow, Univ. of Michigan Hospitals, Ann Arbor, MI

Military Service: None

Faculty Appointments:

1981-1982 Instructor, St. Louis Community College
1992-1993 Lecturer In Internal Medicine, Hematology/Oncology, University of Michigan.
1993-1994 Instructor, Division of Hematology/Oncology, University of Pennsylvania.
1994-pres. Ann B. Young Assistant Professor of Cancer Research, Division of Hematology/Oncology, Department of Medicine, University of Pennsylvania.

Hospital and Administrative Appointments:

1992 Admissions Committee, University of Michigan School of Medicine.
1992-1993 Home Infusion Service, Experimental Therapeutics Grant Review Committee. University of Michigan School of Medicine
1993 Scientific Retreat Committee, Institute for Human Gene Therapy, University of Pennsylvania.
1994-pres. Director of Cancer Gene Therapy Program, Institute for Human Gene Therapy, University of Pennsylvania.
1993-1998 Co-Director, Gene Therapy Program, The University of Pennsylvania Cancer Center
1999-pres. Director, Gene Therapy Program, The University of Pennsylvania Cancer Center

Specialty Certification:

1990 Board Certified, American Board of Internal Medicine
1996 Board Certified, Hematology
1994 Board Eligible, Medical Oncology

Licensure:

Michigan (1989-1996) Pennsylvania (1993-2000)

Awards, Honors and Membership in Honorary Societies:

1972-1975 Heyl Fellowship In Science, Kalamazoo College
 1975 Honors Thesis, Kalamazoo College
 1992-1995 Merck-American Fed. for Clinical Research, M.D./Ph.D. Postdoctoral Fellowship
 1994-pres. Anne B. Young Assistant Professor for Cancer Research, July 1, 1994.
 1995-1996 Measly Fellowship Award
 1998 University of Pennsylvania Nominee for Rita Allen Award.

Memberships in Professional and Scientific Societies:

Local Societies:

Philadelphia Cancer Research Association
 Pennsylvania Chapter of the American Chemical Society

National Societies:

American Association for the Advancement of Science
 American Federation of Clinical Research
 American Association for Cancer Research
 American Chemical Society
 The Brain Tumor Society
 American Society of Gene Therapy

National Scientific Committees

ECOG, Gene Therapy Committee, member	1996-present
NIH, NCI PO1 Review, Boston, MA	6/25-27/95
US Army Breast Can. Res. Program, Ad hoc reviewer	11/13-15/95
Breast Cancer Research Program, University of CA	1996-1998
NCI RFA Review Committee, Ad hoc reviewer	6/11-13/96
NIH, Neurosciences 3 Study Section, Ad hoc reviewer	6/26-28/96
NIH, NCI PO1 Review, New York,	7/7-9/96
NIH, NCI, Medicine Branch, Ad hoc reviewer, Wash. D.C.	9/10-12/96
NIMH PO1 Review, Washington, DC. Ad hoc reviewer,	12/96
NIH, NCI Ad hoc Reviewer	6/30/97
State of Massachusetts Breast Cancer Program	1997, 1998, 2000
North American Brain Tumor Consortium (NABTC) and New Approaches to Brain Tumor Therapy (NABTT)	
consortium multigroup glioma gene therapy clinical trial. Data and Safety Monitoring Committee, Chairman	1998-present
External Reviewer NCI PO1, Massachusetts General Hospital	5/98
NIH, NCI PO1 Review, Los Angeles,	7/27-29/98
State of Massachusetts Breast Cancer Program	10/24-25/98
NCI, Subcommittee D "Clinical Research Studies"	11/30-12/1/98
US Army Ovarian Cancer Study Section	1/20/99-1/22/99
NCI, Subcommittee D "Clinical Research Studies"	4/14-5/99
NCI, RAID Review	3/31/99-4/1/99
NIH, Career Development Award Review	6/21-22/99
NCI, Ovarian Cancer Spore Grants Review	6/27-29/99
NIH, NCI PO1 Review, Durham, NC	1/7/00-1/8/00
National Gene Vector Laboratories (NIH), Scientific Review Board	2000-present
NIH, NCI, Special Emphasis Study Section in Clinical Oncology	4/00 -3/01 (3 times/year)
NIH, NCI, Clinical Oncology Study Section	7/01-present (3 times/year)
NCI, RAID Review	10/1/00
American Society of Gene Therapy, Cancer Gene Therapy Committee	1999-present

Editorial Positions:

Scientific Advisor, Education Committee, Pennsylvania Biotechnology Association, State College, PA 1995
 Cancer Gene Therapy, Editorial Board, Simon & Schuster Publisher 1996-present
 Gene Therapy, Editorial Board, Stockton Press. 1999-present
 Current Gene Therapy, Editorial Board 2000- present
 Ad hoc reviewer for:

Human Gene Therapy, Journal of Immunology, Cancer Research, Journal of Virology, American Journal of Gastroenterology, Gastroenterology, Nature Medicine, Annals of Neurology, Proc. Nat'l Acad. Sciences, DNA and Cell Biology, J. Organic Chemistry, J. Nuclear Medicine, BioTechniques.

Academic Committees at the University of Pennsylvania and Affiliated Hospitals:

Clinical Trials Scientific Review and Monitoring Committee, UPCC 1996-1999
 University of Penn. General Clinical Research Center Internal Review Committee 1996-97
 Faculty Grievance Commission 1997-2000
 Molecular Life Sciences Advisory Committee 1998-present
 Vagelos Scholars Advisory Committee 1998-present
 Short Term Experience in Research Advisory Committee 1999-present
 Office of Human Research Faculty Advisory Committee, School of Medicine 2001-present
 Combined Degree, Cell and Molecular Biology Recruitment Committee

Major Teaching and Clinical Responsibilities at the University of Pennsylvania (last 3 yrs):

1993-1999 Attending Physician, Oncology & Hematology Services, Hospitals of the University of Pennsylvania.
 1994-1999 Attending Physician, Oncology & Hematology Services, Philadelphia Veterans Admin. Hospital.
 1996, 1998, 1999 Human Biology (Biology 6)
 1996 Critical Care Nurse Practitioner Course, "Hematology in the Critical Care Setting"
 1995-1999 Selected Topics in Chemistry (Chemistry 700)
 1996-2000 The Molecular Basis of Gene Therapy, (CAMB 610)
 2000 Medicine 101C, Differential Diagnosis
 1997-1999 Introduction to Gene Therapy (CAMB 610, Fall)
 1999, 2000 Advanced Seminar in Cancer Gene Therapy (CAMB 633, Spring 1999) Course Director
 1997 Wistar Cancer Biology Graduate Student Seminar
 1997, 1998, 2000 Cancer Biology and Genetics Course (CAMB 512, Pathology, Fall)
 1998-2000 Topics in Cancer Pharmacology (PHARM 640 Fall 1998, 1999, 2000)
 1999, 2000 Cancer Pharmacology (PHARM 560)
 2000 Ethics of Human Subjects Research, Medical School Curriculum 2000
 2000 Intro. to Anatomy and Physiology (BSTA 510), A course for Biostatistics Graduate students
 2001 Nuclear Medicine 210
 2001 Frontiers of Pharmacology (FR508) 4th year medical student elective course
 2001 Radiobiology (XXXX) A course for Radiation Oncology Fellows
 2001 "Standard Operating Procedures for Good Clinical Practice" A course for faculty engaged in FDA regulated research
 2001 MD-PHD CLINICAL CONNECTIONS PROGRAM EVALUATION Preceptor

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Summary of Prior Work

- ◆ mEGP when ectopically expressed in BMDC blocks an allogeneic mixed lymphocyte reaction (MLR) as assessed by T cell proliferation, IL-2 production and interferon- γ production.
- ◆ Inhibition of T cell activation by mEGP is dose dependent.
- ◆ T cell activation in the MLR is restored in the presence of mEGP when either Con A, anti-CD3 antibody or SEB super antigen are added to the MLR, however, antibodies to mEGP do not restore T cell responses.
- ◆ mEGP blocks the response of lymphocytes with transgenic T cell receptors for OVA or HEL, either when the intact protein is used as the antigen or when the specific MHC restricted peptides are used as the antigen.
- ◆ mEGP when provided in the form of a lysed cell expressing mEGP is also able to block T cell activation as assessed in both the MLR and OVA model experiments.
- ◆ mEGP does not alter DC morphology, cell surface expression of key T cell stimulatory molecules (e.g., B7-1, B7-2, class I, class II, CD 11b, CD 11c), production of IL-12 and overall DC viability.
- ◆ In vivo administration (intravenous) of DC transduced to express mEGP and adenoviral antigens fails to elicit the expected T and B cell responses to adenoviral antigens.